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Histochemistry and Cell Biology **Short Communication**

Differential expression of PKCα and -β in the zebrafish retina

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Abstract

The retina is a complex neural circuit, which processes and transmits visual information from light perceiving photoreceptors to projecting retinal ganglion cells. Much of the computational power of the retina rests on signal integrating interneurons, such as bipolar cells.

Commercially available antibodies against bovine and human conventional protein kinase C (PKC) α and -β are frequently used as markers for retinal ON-bipolar cells in different species, despite the fact that it is not known which bipolar cell subtype(s) they actually label. In zebrafish (*Danio rerio*) five *prkc* genes (coding for PKC proteins) have been identified. Their expression has not been systematically determined. While *prkcg* is not expressed in retinal tissue, the other four prkc (prkcaa, prkcab, prkcba, prkcbb) transcripts were found in different parts of the inner nuclear layer and some as well in the retinal ganglion cell layer. Immunohistochemical analysis in adult zebrafish retina using fluorescent in situ hybridization and PKC antibodies showed an overlapping immunolabeling of ON-bipolar cells that are most likely of the BON s6 and BON s6L or RRod type. However, comparison of transcript expression with immunolabeling, implies that these antibodies are not specific for one single zebrafish conventional PKC, but rather detect a combination of PKC -α and -β variants.

Introduction

Bipolar cells of the vertebrate retina transmit and shape the light signal from photoreceptors to projecting ganglion cells. One broadly accepted and widely used marker for bipolar cells is protein kinase α (PKCα). First used in the late 1980s (Negishi et al. 1988) antibodies against PKCs soon became popular markers for rod bipolar cells in mammals and the corresponding mixed-type ON-bipolar cell in teleosts (Yazulla and Studholme 1992; Mack 2007)(Greferath et al. 1990; Osborne 1991; Zhang and Yeh 1991; Grünert et al. 1994; Vaquero et al. 1996; Caminos et al. 1999; Haverkamp and Wässle 2000). These cells are presumably labelled by antibodies against PKCα and/or -β (Caminos et al. 2000; Yazulla and Studholme 2001; Connaughton 2011), however it is still unresolved which subset of bipolar cells are in fact labelled.

In the mammalian retina more than ten different subtypes of ON- and OFF-bipolar cells have been identified (e.g. (Famiglietti 1981; Boycott and Wässle 1991; Kolb et al. 1992; Ghosh et al. 2004; Wässle et al. 2009; Macosko et al. 2015). These different subtypes are classified according to their morphology and their connectivity pattern within one or more sublamina of the inner plexiform layer (IPL) (Kolb et al. 1992; Connaughton et al. 2004; Wässle et al. 2009). ON-type bipolar cells typically send their axons to the inner sublamina “b” of the IPL whereas OFF-bipolar cells stratify in the outer sublamina “a” of this layer (Nelson and Kolb 1983; Euler et al. 1996; Saito et al. 1985). Many non-mammalian vertebrates possess a set of mixed-type bipolar cells that send axons to both IPL sublaminae and functionally show both ON- and OFF-response properties (Pang et al. 2004; Wong et al. 2005; Wong and Dowling 2005; Wu et al. 2000). Inter-species comparison is problematic due to the high variability in bipolar cell subtypes and differences in connection patterns. In zebrafish, 17 morphologically distinct bipolar cell subtypes have been described (Connaughton et al. 2004). However, recent studies

considering both axonal stratification pattern and photoreceptor connectivity for the classification of bipolar cells, suggest that the number of different bipolar cell subtypes may even be as high as 33 (Li et al. 2012). Since PKC antibodies are commonly used to label bipolar cells, the detailed expression profile of PKCs and the specificity of these antibodies for each PKC variant are of importance.

PKCα/β belong to the group of conventional PKCs (cPKCs) that consist of the three members PKCα, -β (in two alternatively spliced isoforms I and II), and γ (Newton 2010). cPKCs require diacylglycerol (DAG) along with calcium and a phospholipid such as phosphatidylserine for activation (Steinberg 2008). They play fundamental roles in numerous signal transduction pathways and have been linked to a number of neurological diseases (Sakai et al. 2011), different forms of cancer (Kang et al. 2011), and retinal pathologies such as diabetic retinopathy (Tarr et al. 2013). Due to the whole genome duplication event at the base of the teleost lineage (reviewed in (Glasauer and Neuhauss 2014), more than one gene paralog for *prkca* and *prkcb* exists in zebrafish (Haug et al. 2018). It is currently not known which of these zebrafish orthologs are recognized by the commercially available antibodies and whether there is crossreactivity between the different variants. Moreover, comparative studies about PKC in the retina are missing. The aim of this study is therefore to focus on PKC expression in the zebrafish retina and to correlate the *prkc* transcript expression with the labeling of PKCα and –β antibodies.

Materials and Methods

Fish maintenance and breeding

Adult fish (RRID:ZIRC_ZL84) were maintained under standard conditions at 26 - 28°C in a 14-hour light/10-hour dark cycle. The wild-type strain WIK was used for all experiments described here. Embryos were raised at 28°C in E3 medium (5mM NaCl, 0.17mM KCl, 0.33mM $CaCl_2$, and 0.33mM $MgSO_4$). They were staged according to development in days post fertilization (dpf) (Kimmel et al. 1995). 12 adult fish and more than 500 larvae were used for the experiments. All larval and adult fish used in this study were fixed between 9am and 11am. The fish were euthanized using tricaine (ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich, Buchs, Switzerland) and iced water. All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local authorities (Veterinäramt Zürich TV4206).

(Fluorescent) *in situ* hybridization ((F)ISH)

Primers used for the generation of RNA probes were used as published (Haug et al. 2018). The plasmids containing cDNA sequences of the different *prkcs* were linearized with the appropriate restriction enzymes, and the DNA was extracted with a standard phenol/chloroform protocol using pre-spinned RNase-free Phase-Lock tubes (5 Prime, Hamburg, Germany). Linearized DNA was *in vitro* transcribed using the DIG-RNA labeling kit (Roche, Rotkreuz, Switzerland), and applied on adult zebrafish retinal sections as previously described (Haug et al. 2018) at a concentration of approximately 2 ng/µl. FISH was performed as described in (Huang et al. 2012), however, fluorescent labeling was accomplished using the TSA kit #12 (Molecular Probes, Life Technologies, Zug, Switzerland). Images were taken by confocal

microscopy (CLSM SP5 and TCS LSI, Leica, Heerbrugg, Switzerland), z-stacks that covered a depth of 1 – 2 µm were selected and processed using ImageJ (Version 1.49, August 2014, Java), and further processed (brightness, contrast and gamma levels of the whole image) and arranged with Adobe Photoshop (RRID:SCR_014199) and Illustrator CS5.

Immunohistochemistry

Zebrafish tissue was prepared similar as described in a previous publication (Haug et al. 2013). Multiple sections from minimally 72 larvae were processed. Briefly, tissue was fixed for 40 min in 4% paraformaldehyde in 1× PBS, washed with 1× PBS, and incubated overnight in 30% sucrose at 4°C. Tissue was embedded in Tissue Tec medium (Sakura Finetek Europe) and directly frozen in liquid nitrogen. Sections of 16 µm were cut using a cryotome, dried for 30 min at room temperature, and stored at −80°C. Immunohistochemical labeling was performed as previously described (Fleisch et al. 2008), with the following modifications: Blocking solution contained 0.3% Triton X-100 (Sigma-Aldrich) instead of Tween 20 and was applied for 30 min at RT. Different primary anti-PKCα and –β antibodies were used at the following concentrations: PKCα NBP1-19273, 1:500 (Novus, Abingdon, UK); PKCα MC5, NB 200-568, 1:1000 (Novus); PKCα MC5, 1:1000 (Genetex), PKCβ1 C16, SC209, 1:1000 (Santa Cruz Biotechnology, Heidelberg, Germany). Anti-rabbit and anti-mouse Alexa 488 (1:1000; Roche) and/or Alexa 568 (1:500; Roche) were used as secondary antibodies. Slides were mounted with Mowiol-DABCO mounting medium (10% Mowiol 4-88 (Polysciences, Warrington, USA), 25% glycerol, 2.5% DABCO (1,4-diazobicyclo[2.2.2]octane, Sigma-Aldrich) in 100 mM Tris–HCl pH 8.5) and stored in darkness at 4°C. Images were taken by confocal microscopy (CLSM SP5 and TCS LSI, Leica, Heerbrugg, Switzerland) and arranged in Figure 1. Images a-a'' and b-b'' cover a z-stack of 4.6 and 4.8 µm, respectively, images c-c'' measure 1.6 µm in depth,

and images d and e are only composed of one single plane. All images were selected and processed using ImageJ (Version 1.49, August 2014, Java), and further processed (brightness, contrast and gamma levels of the whole image) and arranged using Adobe Photoshop (RRID:SCR_014199) and Illustrator CS5.

Expression of recombinant PKCs and Western blot

The coding region of each zebrafish *prkc* was amplified using the primers listed in Table 1 and subcloned into pIRES2:EGFP vector (kindly provided by M. Kamermans). Expression was done in HEK293T cells. Transient transfection of cells using the $Ca_3(PO_4)_2$ technique was performed as previously described (Jordan et al. 1996). 5 µg linearized DNA or buffer (mock transfection) was added to the cells. 30 to 40 hours post-transfection when the cell layer had reached a confluence of up to 100%, cells were checked for GFP signals. Subsequently, the medium was aspirated and the plates were placed on ice and washed three times with 3 ml cold PBS containing 0.9 mM $CaCl_2$. Next, cells were lysed by adding 500 µl Laemmli buffer (4.4 ml 0.5M Tris-HCl pH 6.8, 4.4 ml Glycerol, 2.2 ml 20% SDS, 0.65 ml 1% Bromophenol blue) supplemented with Protease inhibitor (Complete Mini, Roche), and collected in a 2 ml Eppendorf tube. Cell lysates were supplemented with 1:40 β-Mercaptoethanol and homogenized with a pistil. After heating them to 90°C for 5 min, the lysates were cleared using centrifugation, the supernatant sonicated and subjected to Western blot analysis. Lysates were loaded on a 10% precast gel (Mini Protean TGX, Biorad, Cressier, Switzerland), blotted to PVDF membranes (0.2 µm, Novex, Thermo Fisher Scientific) which were blocked for 2 hours in PBS containing 0.05% Tween 20 and 3% dry milk powder (PBS-TM) at RT. Primary antibodies were used at the same concentrations as described for immunohistochemistry and applied over night at 4°C in PBS-TM. As a loading

control anti-Vinculin (124 kDa; 1:5000, Genetex) was used. After a 5 min washing step in PBS-TM followed by two 10 min washing steps in PBS containing 0.05% Tween 20 (PBT), the membranes were incubated for 45 min at RT with secondary horseradish peroxidase (HRP-) linked antibodies (Invitrogen, Thermo Fisher Scientific) diluted in PBS-TM (goat anti-rabbit, 1:5000; goat anti-mouse, 1:7500). Following a 20 min washing with PBS-TM and four 5 min washes with PBT, membranes were subjected to development solution (Super Signal West Dura Extended Duration Substrate, Thermo Fisher Scientific) for 5 min at RT. Finally, the signals were detected by the LAS 4000 Chemiluminescence Imager (software: Image Quant LAS 4000, automatic exposure) and processed using Adobe Illustrator C5.

Results

In mammals, the family of conventional *prkcs* consists of the three members *-a*, *-b* and *-g* (Newton, 2010). Based on sequence similarity, we annotated and cloned five different zebrafish *prkc* cDNAs, two paralogs of *prkca* and *prkcb* and one single *prkcg* paralog. The phylogeny and the detailed description of the larval expression pattern of these genes is reported in (Haug et al. 2018). In this study, we describe *prkc* transcript expression in the retina by *in situ* hybridization in combination with PKC antibody labeling using commercially available antibodies.

Commercially available PKCα and -β antibodies label overlapping subsets of ON-bipolar cells

PKCα and -β antibodies are used as markers for retinal ON-bipolar cells in different species (Yazulla and Studholme 1992; Mack 2007)(Greferath et al. 1990; Osborne 1991; Zhang and Yeh 1991; Grünert et al. 1994; Vaquero et al. 1996; Caminos et al. 1999; Haverkamp and

Wässle 2000; Yazulla and Studholme 1992). As there are no PKC antibodies specifically raised against zebrafish peptides or proteins, commercial PKC antibodies are commonly used as markers for bipolar cells. We now tested different frequently used PKC antibodies raised against bovine and human epitopes and found marked differences in their staining profile of zebrafish bipolar cells (Fig. 1).

In zebrafish all used PKC antibodies showed specific labeling in the retinal INL and IPL, presumably in ON-bipolar cells and their processes (Fig. 1 panels a, b, c). A separate double labeling of each PKCα MC5 with PKCβ showed that all antibodies label identical cells in the middle part of the INL (Fig. 1a'-'' and b'-''), some with smaller axon terminals (arrows) and some with a larger axon terminal (arrowheads). In addition, both PKCα MC5 antibodies stain structures in the GCL (asterisk in Fig. 1a'',b''). Another PKCα antibody (Novus (NBP)) also weakly labels the same cells in the INL but showed a very strong labeling in the retinal ONL, presumably in accessory outer segments (Fig. 1c'-'') (Hodel et al. 2014). Aside from the retina, PKC antibodies additionally label different cells in other tissues. Applying PKCα MC5 (Genetex) and PKCβ on transverse sections of the brain and the jaw of 5 days old zebrafish larvae shows antibody-specific labeling in distinct areas of both examined tissue samples (Fig. 1d,e), demonstrating that these antibodies recognize different zebrafish PKCs with different affinities. Hence, the labeled ON-bipolar cells might express a mix of different PKCs.

prkc transcripts in the zebrafish retina are expressed in overlapping but distinct patterns

To gain a detailed view of *prkc* expression in the zebrafish retina, we analyzed adult retinal tissue by *in situ* hybridization (ISH). While both paralogs of the zebrafish *prkca* and *–b* genes are expressed in the adult zebrafish retina, we never observed expression of *prkcg* (Fig. 2a,c,e,g,). Therefore, we excluded *prkcg* from further analysis. In contrast to *prkcaa* mRNA

that can be detected in the middle INL (Fig. 2a), *prkcab* and the two *prkcb* transcripts are more widely expressed. *prkcab* is expressed in the middle and the distal INL, as well as in the GCL (Fig. 2c). A strong labeling in the proximal INL and the GCL is seen for *prkcba* (Fig. 2e), whereas *prkcbb* is only weakly expressed throughout the INL and in the GCL (Fig. 2g). The expression pattern using the same probe but with a fluorescent tag (FISH) were generally overlapping (Fig. 2 b1,d1,f1,h1). For *prkcbb* we found an additional weak expression in the ONL (arrowhead in Fig. 2h1), suggesting a difference in the sensitivity of the two detection methods.

PKCα MC5 antibody labeling highlights prkcαa, –αb, and –βb expressing cells and the corresponding proteins

As the different zebrafish *prkcs* were not expressed in the same retina layers, we combined *prkc* fluorescent RNA labeling with antibody staining in adult retinal sections to demonstrate which *prkc* expression overlaps with the PKC antibody labeling. We chose to use PKCα MC5 as a marker for this experiment, as it shows an overlapping labeling in bipolar cells with all other antibodies tested but also labeling in some additional cells in the GCL compared to PKCβ (Fig. 2a'').

Interestingly, all PKCα-positive bipolar cells clearly express *prkcaa* within their cell bodies and vice versa (Fig. 2b3-4), whereas the *prkcab* transcript seems to be located in some but not all bipolar cells labeled by the PKCα antibody (Fig. 2d3-4). For *prkcb* genes we found no overlap of the antibody labeling with *prkcba* (Fig. 2f3-4) and only a partial overlap with *prkcbb* (Fig. 2h3-4).

In order to gain insight into PKC antibody specificity in zebrafish we generated expression constructs of full-length *prkc* transcripts, and tested antibody recognition of recombinant

proteins by Western blot (Fig. 3). Since both PKCα MC5 antibodies showed comparable results, only the result with the PKCα MC5 from Genetex is shown. For each antibody, a different pattern can be observed (see overview in Fig. 3d). When applying the PKCα NBP antibody on recombinant zebrafish PKCs, bands in different intensities around the expected size of 75 kDa can be detected (Fig. 3a). In addition to that, the PKCα NBP antibody recognizes a faint band of a lower size for PKCβa (black arrowhead in Fig. 3a, 3rd lane) and a strong band at a higher position for PKCβb (white arrowhead in Fig. 3a, 4th lane). The PKCα MC5 antibody recognizes the zebrafish PKCαb at exactly 75 kDa (Fig. 3b, 2nd lane), and in addition PKCαa and PKCβb at a slightly higher position (Fig. 3b, 1st and 4th lane). The PKCβ1 antibody strongly recognizes recombinant zebrafish PKCαa (Fig. 3c, 1st lane) and weakly PKCβb (Fig. 3c, 4th lane), but none of the other recombinant PKCs (Fig. 3c, 2nd, 3rd, 5th and 6^{th} lane). These western blot results confirm that these antibodies recognize various antigens, explaining their differential immunohistological labeling of differing bipolar cell populations.

Discussion

After analyzing the expression and phylogenetic relations of zebrafish *prkc* genes (Haug et al. 2018) we focused on the retina, as PKCα and –β antibodies are widely used in the community as markers for ON-bipolar cells. We performed fluorescent ISH in combination with PKCα antibody labeling and Western blots using recombinant PKCs to identify which zebrafish PKC(s) are labeled by the commercially available antibodies.

PKC antibodies recognize different zebrafish PKC variants

Although non-mammalian vertebrates possess a significantly higher diversity of bipolar cells than mammals, their main functions are conserved (Euler et al. 2014). PKCα and sometimes PKCβ antibodies are commonly used as ON-bipolar cell markers. However, the frequently used antibodies against PKCα and/or -β have been designed to recognize human or bovine epitopes. Hence, it is not known which PKC proteins are recognized by the commonly used antibodies. Moreover, the whole genome duplication event in the lineage of teleost fish has added to the complexity (Glasauer and Neuhauss 2014), increasing the number of zebrafish *prkcs* to five. This increase in retinal genes may be the basis for the 33 different types of bipolar cells that were recently identified in zebrafish (Li et al. 2012). Another explanation could be that some aspects of visual processing that takes place in higher brain areas of mammals is achieved in the retina of lower vertebrates.

Our initial ISH showed labeling of both *prkca* and *prkcb* paralogs in the INL of the retina where bipolar cells are located. We found a complete overlap in cells of the middle INL when applying the riboprobe of *prkcaa* and the antibody against PKCα MC5 (Fig. 2b1-4). Some but not all PKCα-positive cell bodies did also express *prkcab* and *prkcbb* (Fig. 2d1-4, h1-4), indicating that at least some PKCα-positive cells express different *prkc* paralogs, while others

only seem to exclusively express *prkcaa*. Interestingly, while the PKCα MC5 antibody indeed recognizes the zebrafish PKCαa, it also recognizes other zebrafish PKCs (Fig. 3). Moreover, western blot results indicated that all tested PKC antibodies recognize a combination of PKCs, demonstrating that the tested antibodies are not specific for one single zebrafish PKC but rather label a mixture of different PKC subtypes. When tested on other tissues such as the brain and the jaw of 5 day old larvae, the PKCα MC5 and the PKCβ antibodies were expressed in clearly different areas, indicating that these two antibodies indeed do not recognize the same combination of zebrafish PKCs as also shown by Western blots (Fig. 3).

PKCα and PKCβ antibodies as marker for ON-bipolar cells

It is generally assumed that PKCα (Caminos et al. 2000) or PKCα/β antibodies (Yazulla and Studholme 2001) stain B_{ON} s6L cells, a bipolar cell type morphologically resembling the mixed-input (b1 or Mb) ON-bipolar cells of other teleosts (Connaughton et al. 2004). Recent data suggest that the B_{ON} s6L bipolar cell is identical to the RRod cell, an ON-bipolar cell that only contacts rods (Li et al. 2012). However, earlier studies describe labelling of an additional bipolar cell type by PKCα or PKCα/β antibodies (Suzuki and Kaneko 1990; Yazulla and Studholme 2001), which possibly corresponds to the slightly smaller B_{ON} s6 type that contacts cones (Connaughton et al. 2004).

All tested antibodies labeled the same cells of the INL in adult zebrafish retina and they seem to be of at least two kinds: We find labeled cells that contain large axon terminals in a more proximal part of the IPL as well as other cells with smaller axon terminals that ramify in a more distal part of the IPL. Based on morphology, these two subtypes likely comprise the above mentioned B_{ON} s6L or RRod type and the B_{ON} s6 type (Connaughton et al. 2004; Li et al. 2012).

As *prkcaa* expression and the antibody labeling are identical, this indicates that *prkcaa* is expressed in these two different bipolar cell subtypes as well.

The second *prkca* paralog, *prkcab*, is also expressed in the middle INL, however its expression only partially overlaps with PKCα-positive cells. This different expression suggests a gain of function after the teleost-specific whole genome duplication (Force et al. 1999; Glasauer and Neuhauss 2014). Interestingly, *prkcbb* is also located in the middle INL and overlaps with some PKCα-positive cells, however, it might also label additional subtypes. However, there remains a discrepancy between the MC5 antibody staining, the fluorescent in situ hybridization and the results obtained from western blotting experiments. While in western blots the MC5 antibody recognizes PKCαa, PKCαb as well as PKCβb, in the retina not all cells expressing transcripts for these proteins are recognized. There are a number of possible explanations to account for this apparent difference: First, as PKCs sometimes come in different splice variants (as has been documented for human *prkca* and zebrafish *prkcba),* the MC5 antibody might not recognize all these variants, while the longer *in situ* probe does not discriminate between these splice variants. Second, cellular localization of the transcripts and the proteins might not be identical, as transcripts are generally found in the cell body, whereas proteins could locate to all different compartments within the cell, therefore making colocalization studies by fluorescent in situ hybridization and antibody staining difficult to compare.

Intriguingly, in larval tissue both *prkca* paralogs label cells in the middle INL (Haug et al. 2018) but expression in other retinal cells is only visible in adult sections (Fig. 1). In addition, both *prkcb* paralogs are only expressed in the adult retina (Fig. 1) (Haug et al. 2018). For most species it is hypothesized that PKCα and/or -β labels additionally rod ON-bipolar cells (Euler and Wässle 1995; Greferath et al. 1990; Negishi et al. 1988; Osborne et al. 1991) and our study also indicates the labeling of RRod cells. As the rod circuitry is established only at later stages

and has been shown to be functional earliest in 15 day old larvae (Bilotta et al. 2001) expression of molecules involved in rod ON-bipolar cell signaling are expected to appear only in older larvae. Taken together our results suggest that PKC variants are also expressed in RRod cells and that the commercial antibodies recognize at least a subset of these cells.

prkc expression in other retina cells

Besides the expected expression in bipolar cells, both *prkca* and *-b* paralogs were also found in additional retinal cell types. So far, studies describe the expression of conventional PKCs in photoreceptors (Ohki et al. 1994; Osborne et al. 1991; Osborne et al. 1992; Udovichenko et al. 1993; Usuda et al. 1991), and in rod outer segments (Kapoor and Chader 1984; Udovichenko et al. 1996). However, other studies were unable to detect PKC-positive photoreceptors (e.g. (Greferath et al. 1990; Kolb et al. 1993; Negishi et al. 1988) or show ambiguous results depending on the applied technique (Kosaka et al. 1998; Williams et al. 1997) or the species examined (Osborne et al. 1991; Caminos et al. 2000). While we did not detect any *prkc* transcripts in photoreceptors by conventional ISH, fluorescent ISH of *prkcbb* showed a weak expression in the ONL suggesting that low concentration of transcripts are indeed present. This is in line with reports about the crucial role of PKCs in photoreceptor development (Pinzon-Guzman et al. 2011) as well as phosphorylation of a number of molecules important for phototransduction (e.g. rhodopsin) (Greene et al. 1997; Udovichenko et al. 1997; Wood et al. 1997). Interestingly, the PKCα NBP antibody (Fig. 1c',c'') shows a similar expression in the photoreceptor layer as was previously published by Osborne and colleagues for a PKCα labeling in the rabbit retina (Osborne et al. 1992). As the company states on the product datasheet that this antibody recognizes the PKCα, –β, and –δ isoform, the labeling in photoreceptors might be due to any of these PKCs.

prkcab, *-ba* and *-bb* all show an additional expression in the INL besides the expected expression in bipolar cells. Due to the location in the distal or proximal INL, respectively, we assume *prkcab* to be expressed in horizontal cells and *prkcba* in amacrine cells while *prkcbb* is distributed throughout the INL and might be expressed in both retinal cell types. Earlier investigations have shown an involvement of PKCs in activity dependent morphological changes of horizontal cell synapses (Rodrigues and Dowling 1990; Weiler et al. 1991), however, this was disputed later (Schmidt 1996). Vertebrate amacrine cells, however, do definitely also express conventional PKCs. This was shown by subtype-specific labeling of PKCα in rat and rabbit (Kosaka et al. 1998; Usuda et al. 1991), and PKCβ in rat and human (Kolb et al. 1993; Kosaka et al. 1998). As we only found evidence for *prkcba* and maybe *prkcbb* expression in the proximal INL where amacrine cells are located, one of the PKCβ-paralogs might cover the function of the PKCα in these cells in zebrafish.

Interestingly, at least in the rodent retina PKCγ has also a function, as the lack of PKCγ (but also of PKCβI) totally inhibits rod development in mice (Pinzon-Guzman et al. 2011). Previous studies are conflicting with some reporting expression of PKCγ in the retina (Kolb et al. 1993; Osborne et al. 1992), while others failed to detect any labeling (Fukuda et al. 1994; Kosaka et al. 1998; Usuda et al. 1991; Wang et al. 2014). In our study, the absence of retinal expression of *prkcg* cannot be attributed to technical issues, since the *prkcg* probe shows distinct cerebellar expression (Haug et al. 2018).

We find a weak *prkcab* and *–bb* expression as well as a very pronounced expression of *prkcba* in the retinal ganglion cell layer of adult zebrafish, however, in an earlier study we did not detect any *prkc* expression in the GCL of larval tissue (Haug et al. 2018). This is in accordance with previous studies, where PKCβ has been located in the GCL (Fukuda et al. 1994; Kolb et

al. 1993; Kosaka et al. 1998; Osborne et al. 1992; Usuda et al. 1991) but only few studies describe the expression of PKCα in this retinal layer (Kolb and Zhang 1997; Wang et al. 2014). In line with the transcript expression, we find the PKCα MC5 antibody of both companies to label cells in the GCL (asterisk in Fig. 1a'', b''), indicating that these antibodies recognize at least one of the β-paralogs as well. (Yazulla and Studholme 2001)

Conclusion

Commercial PKCα and –β antibodies are commonly used to label ON-bipolar cells in the vertebrate retina. We found that these antibodies, indeed as previously suggested, consistently label a subset of ON-bipolar cells of both the scotopic and photopic pathway (B_{ON} s6L or RRod type and B_{ON} s6) in the zebrafish retina. However, these antibodies are neither, as often considered, pan-ON-bipolar cell markers, nor are they specific for a single PKCα or PKCβ variant but rather mark different bipolar cell subtype populations and recognize multiple PKC paralogs.

Competing interests

We have no competing interests.

Authors' contributions

MFH carried out the experiments, participated in the design of the study and drafted and edited the manuscript; MB helped with the *in situ* hybridization and the fluorescent *in situ* hybridization, and drafted the manuscript; MG participated in the design of the study and edited the manuscript; SCFN participated in the design of the study, coordinated the study and edited the manuscript. All authors gave final approval for publication.

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Table 1: Primers used for the cloning of expression constructs

Table 1: Primer sites used for the cloning of expression constructs.		
Gene	**Sequence 5‘-3‘**	**Linearization**
prkcaa_fwd	AAAAAGCTAGCGCCACAATGGCTGATACACAAAG	NheI - BglII
prkcaa_rev	TTTTTAGATCTTTCCCCTTTTTTATTC	
prkcab_fwd	AAAAAGAATTCGCAACCATGGCTGATCATCTGATACA	EcoRI SalI
prkcab_rev	TTTTTGTCGACTCCTGGGACGTCTCATAC	
prkcba_fwd	AAAAAGAATTCCCTATCATGACCGAGTC	EcoRI - SalI
prkcba_rev	TTATTGTCGACTTGGCTAAAACTGGCTAC	
prkcbb_fwd	AAAAACTCGAGCGCAGAATGGCAGAGCCGG	XhoI - BamhHI
prkcbb_rev	TTTTTGGATCCGGTCGCCCTTAACTCTG	
prkcg_fwd	AAAAACTCGAGTCAAACATGGCTGGTCTGGACCCTGG CGTAGGCGATTCAGAAGGTGGACCCCGGCCTCTGTTT TGCAGGAAAGGAGCTCTCAAGC	XhoI - SalI
prkcg_rev	TATATGTCGACTGAAATTGGTATGTGTGAACTG	

Table 2: Overview of the antibodies used in this study

Antigen	**Description of Immunogen**	**Source, Host species, Cat#, Clone or Lot#, RRID**	**Concentration used**
PKCα [MC5]	Purified bovine brain protein kinase C alpha	Novus, mouse, monoclonal, Cat# NB 200-586, Clone# MC5, AB_2252787	1ug / 1ml
PKCα [MC5]	Purified bovine brain protein kinase C alpha	Genetex, mouse, monoclonal, Cat# GTX20031, Clone# MC5, AB_384212	2.7ug / 1ml
PKCα	Synthetic peptide derived form the sequence of human PKC, conjugated to KLH, sequence identical between the alpha, beta and delta isoforms of PKC.	Novus, rabbit, polyclonal, Cat# NBP1-19273, AB_1642848	1:500 from original tube, no information about concentration provided
cPKCβ1 C16	Synthetic peptide corresponding to amino acids 656-671 at the C-terminus of PKCβI of human origin (Breuiller-Fouché et al.,1998)	Santa Cruz, rabbit, polyclonal, sc-209	0.2ug / 1ml

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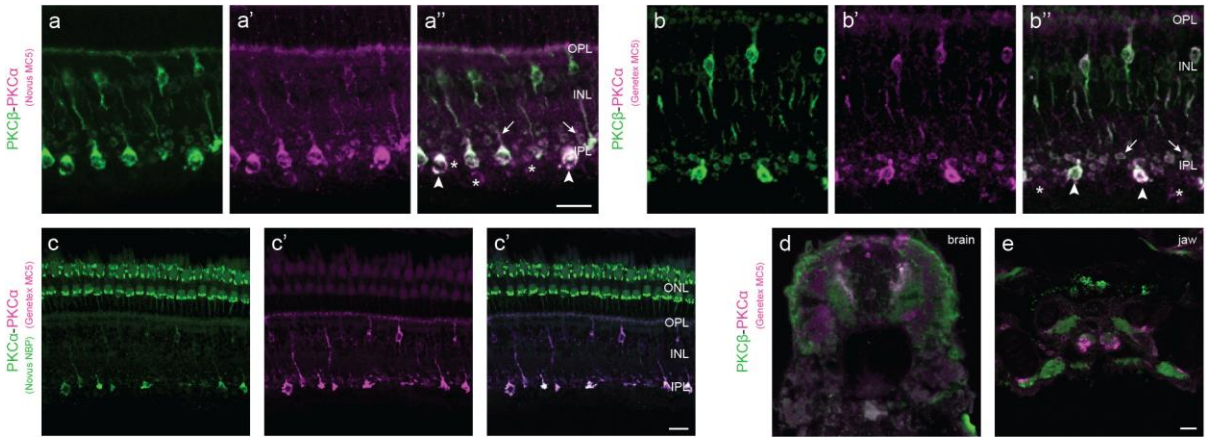

Figure 1: Commercially available PKC antibodies label overlapping but also distinct central nervous system structures

Double labeling of PKCα (MC5 Novus, MC5 Genetex, and NBP Novus) and PKCβ (Santa Cruz Biotechnology) antibodies on retinal sections of adult (a-c) and 5 dpf larval (d,e) zebrafish. Both PKCα MC5 antibodies show an overlapping labeling in retinal bipolar cells with PKCβ (a'' and b''). Different shapes of axon terminals in the IPL (arrows and arrowheads in a'' and b'') suggest labeling of at least two different bipolar cells types. In addition, with both PKCα MC5 antibodies a weak labeling of some cells in the GCL (asterisk in a'' and b'') can be detected. A third PKCα antibody (NBP, Novus) is expressed in an overlapping manner in the INL and IPL but labels in addition accessory outer segments of photoreceptors in the ONL (c-c''). Double labeling on transverse sections of 5 dpf larvae shows that the PKCα (MC5, Genetex) and –β antibodies additionally label different areas of the brain and the jaw (d,e). For abbreviations, see list. Scale bar in a'' (applies to a'-'' and b'-''), in c'' (applies to c'-'') and in e (applies to d and e) = 10 µm.

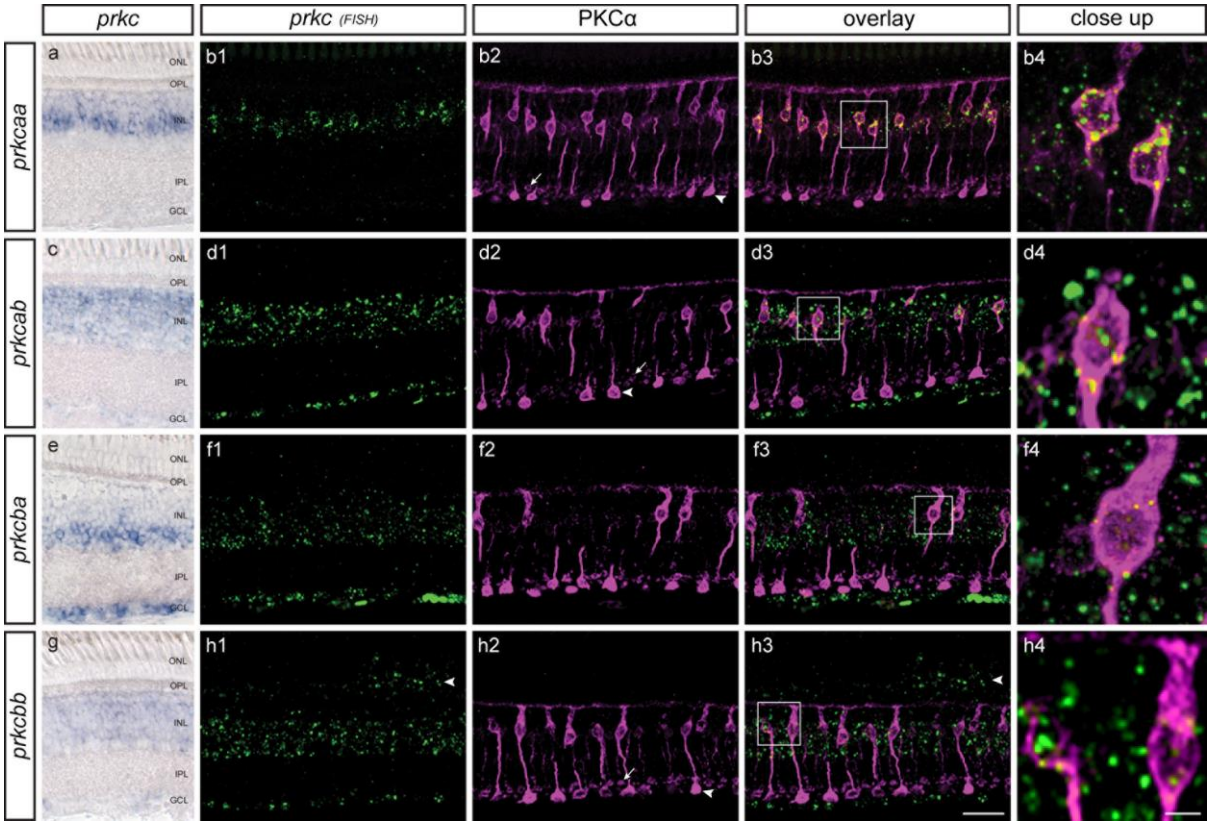

Figure 2: Co-expression analysis of *prkca* and *–b* transcripts with PKCα antibody labeling in adult retinal sections

Conventional (blue) and fluorescent (green) mRNA *in situ* hybriziation of *prkca* and *–b* paralogs (a,c,e,g and b1,d1,f1,h1). Both *prkca* paralogs are expressed in the inner nuclear layer (INL) of the retina. While the expression of *prkcaa* is restricted to the middle INL (a, b1), *prkcab* transcripts show a broader expression in the middle and the distal INL, and in the ganglion cell layer (GCL; c, d1). *prkcba* is expressed in the proximal INL and strongly in the GCL (e, f1), and its paralog, *prkcbb*, weakly throughout the INL and GCL (g, h1). Additionally, photoreceptors are weakly labeled by the fluorescent method (asterisk in h1).

Confocal images of adult retinal sections showing fluorescent *in situ* hybridization (green) in combination with PKCα MC5 antibody labeling (magenta) and the corresponding overlay (b3,d3,f3,h3). PKCα labeling is found in two different types of bipolar cells, as illustrated

with arrows vs. arrowheads. We find expression of *prkcaa* in all PKCα-positive bipolar cell bodies in the middle INL (b3-4), while *prkcab* and *–bb* show only partial overlap (d3-4, h3-4). *prkcba* transcripts seem not to overlap with PKCα-positive cells (f3-4). For abbreviations, see list. Scale bar in h3 (applies to all images except the close ups) = 25 µm. Scale bar in h4 (applies to b4,d4,f4,h4) = 5 µm.

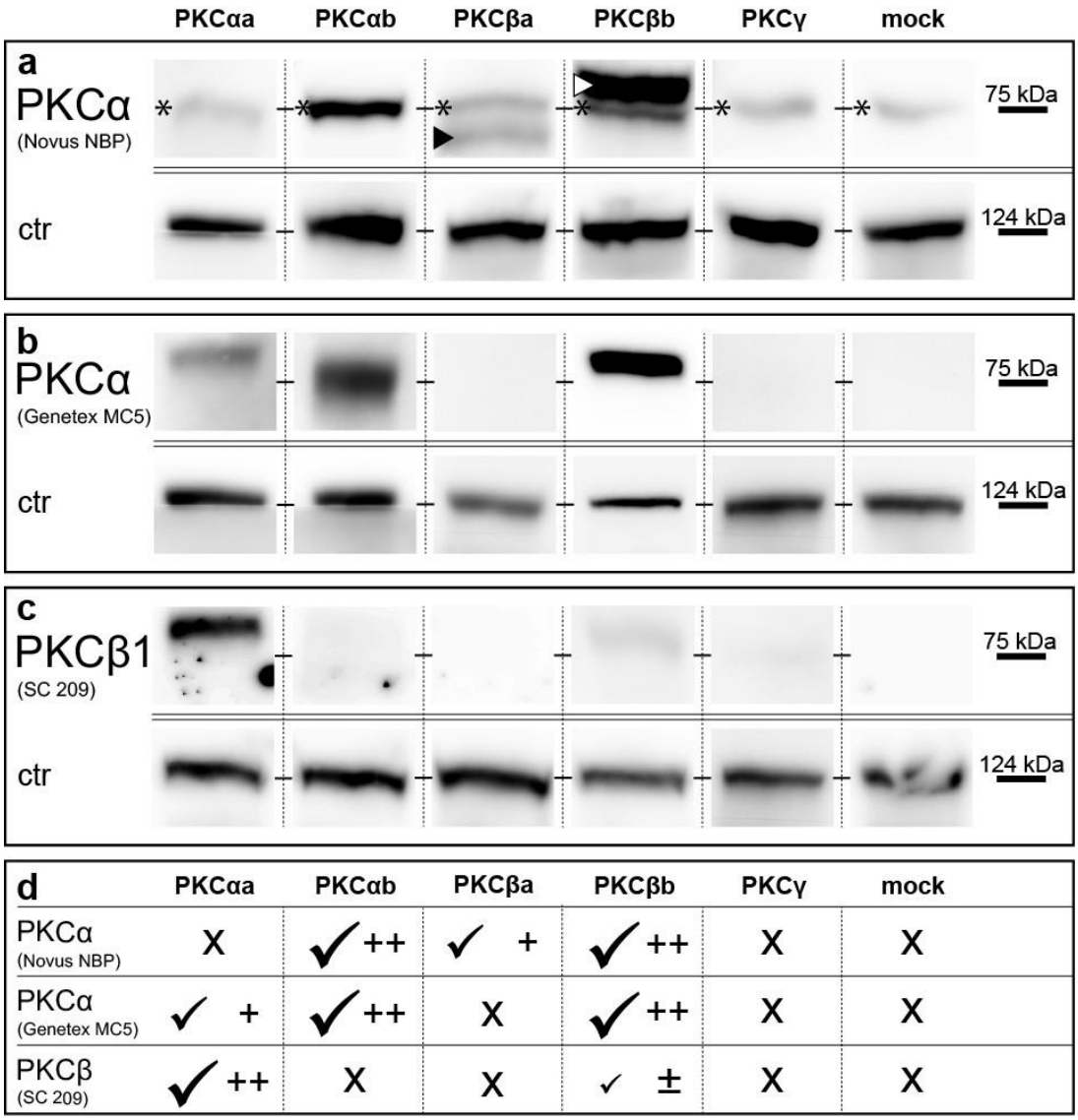

Figure 3: Western blot analysis of recombinant zebrafish cPKC proteins using PKCα and –β antibodies

Three different antibodies were used to compare the specificity of each antibody with recombinant zebrafish PKCs. The PKCα NBP antibody are specific for PKCαb and both PKCβ paralogs. PKCα NBP detects a faint band in all samples, including mock controls, likely recognizing an endogenous distantly related PKC orthologue present in HEK293T cells (asterisk at 75 kDa), PKCα MC5 recognizes both zebrafish PKCα paralogs (b, 1. and 2. lane) as well as PKCβb (b, 4. lane). The PKCβ1 antibody also recognizes PKCαa (c, 1. lane) but

PKCβb only faintly (c, 4. lane), and PKCαb not at all (c, 2. lane). PKCγ lysates were not specifically detected with any antibody used (a-c, 5. lane). Anti-Vinculin (124 kDa) antibodies were used as loading controls. The different antibody recognition patterns are summarized in panel d.